The present amendment responds to the issues raised in the final action in the pending application. A single remaining rejection is of record – a § 103 rejection based on the combination of the Pain et al. (1996) paper and the Gibbins et al. (1990) reference. The withdrawal of the rejection is necessary because the Examiner's interpretation of the Pain et al. and Gibbins et al. references on which the rejection is based, is demonstrably in error. Furthermore, the Examiner has asserted the existence of long-term cell cultures and the existence of chimeric birds that encode a transgene. No such composition or animal exists in the prior art and this basis for the § 103 rejection cannot be maintained.

The sole remaining rejection to the pending claims is based on the Examiner's reliance on an isolated statement in Pain et al (1996) that: "Regardless of the number of passages, more than 50% of the hatched recipient embryos were chimeras with nearly 33% of the plumage from donor phenotype". (page 2344 col 2 para 2). This is clearly a misquotation because the statement modifies the sentence above which states "Cells were collected from cultures after 1-3 passages". Hence, the meaning of the sentence is clearly that within the first three passages, chimeras could be produced. The final statement of the paragraph says: "The ability of long-term cultures to give rise to chimeric animals is currently under investigation". Clearly, chimeras could not be made from cells that had been in culture for longer than 19 days as is explicitly stated in the caption to Figure 8 of the reference.

Furthermore, because the legend for Fig. 8 says "...chimeric chicks derived from White Leghorn embryos grafted with Barred Rock CEC cultivated during 3 to 19 days," there can be no evidence that chicken ES cells could be cultivated for periods sufficient long to establish a stably transfected line of ES cells. Again, the final statement of the paragraph says: "The ability of long-term cultures to give rise to chimeric animals is currently under investigation." At that time, chimeras could not be made from

cells that had been in culture for long periods. The art does not contain any example of ES cells stably transfected with a transgene.

To summarize, the Pain et al (1996) paper teaches a culture containing cells which could produce chimeras, within 3 passages, when maintained in culture for 19 days or less -- characteristics that are shared with mouse ES and EC cells. For a short period of time (i.e. up to 19 days) they would make somatic chimeras and for 7 days they would make germline chimeras. However, after longer periods in culture, they would make neither somatic nor germline chimeras. All of these attributes are described in the paper and stand in sharp contrast to the interpretation of the examiner.

Gibbins et al. (1990) does not disclose selection of stably transfected cells nor the production of chimeras from these cells. Gibbins et al. (1990) specifically discloses that the goal has yet to be achieved. Gibbins et al. (1990) state:

> "A major problem that we have encountered is that we have not been successful in culturing chicken embryonic stem cells for any extended period of time without differentiation taking place."

Thus, genetically modified stem cells necessary to make the invention do not exist and the Examiner's proposition that the teaching of Pain establishes ES cells genetically modified stem cells (per Gibbins) cannot stand because Gibbins did not, and could not, produce genetically modified cells because the culture could not be maintained "for any extended period of time" such that transfection and selection take place.

As recently as 2004, researchers in the field of avian pluripotent stem cells described attempts to make chimeras from embryonic stem cells in culture and did not report the creation of chimeras from any culture longer than 20 days. Petitte et al. *Mechanisms of Development 121:* 1159-68 (2004) (attached), see Table 1.

The Examiner cannot legitimately contend that a reasonable expectation of success exists when the entire body of prior art cited by applicants and the Office reveal numerous failed attempts to create chimeras from cells held in long-term culture and do not reveal a single example beyond 20 days – even as recently as this year. The claimed method is the only successful creation of a chimera from a cell culture greater than 60 days old.

The Examiner also cannot maintain that the claimed chimeric chicken is indistinguishable from the prior art, because prior art chimeras do not have a stably integrated transgene. The Examiner has not, and cannot, cite an example in the prior art of a chimera having a transgene because none existed prior to the present invention.

The Examiner's rejection of the facts established by the Etches declaration cannot stand because the rejection is based on the same misinterpretation of the Pain et al. paper. The statement that Pain et al "clearly teaches in-vivo differentiation of chicken embryo cells ... for up to 60 days[.]" is directly contradicted by the express language of the paper where the maximum length of 19 days is disclosed.

Applicants contend that the pending claims are in condition for allowance and request such action accordingly.

Respectfully submitted,

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Review

Avian pluripotent stem cells

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Abstract

Pluripotent embryonic stem cells are undifferentiated cells capable of proliferation and self-renewal and have the capacity to differentiate into all somatic cell types and the germ line. They provide an in vitro model of early embryonic differentiation and are a useful means for targeted manipulation of the genome. Pluripotent stem cells in the chick have been derived from stage X blastoderms and 5.5 day gonadal primordial germ cells (PGCs). Blastoderm-derived embryonic stem cells (ESCs) have the capacity for in vitro differentiation into embryoid bodies and derivatives of the three primary germ layers. When grafted onto the chorioallantoic membrane, the ESCs formed a variety of differentiated cell types and attempted to organize into complex structures. In addition, when injected into the unincubated stage X blastoderm, the ESCs can be found in numerous somatic tissues and the germ line. The potential give rise to somatic and germ line chimeras is highly dependent upon the culture conditions and decreases with passage. Likewise, PGC-derived embryonic germ cells (EGCs) can give rise to simple embryoid bodies and can undergo some differentiation in vitro. Interestingly, chicken EG cells contribute to somatic lineages when injected into the stage X blastoderm, but only germ line chimeras have resulted from EGCs injected into the vasculature of the stage 16 embryo. To date, no lines of transgenic chickens have been generated using ESCs or EGCs. Nevertheless, progress towards the culture of avian pluripotent stem cells has been significant. In the future, the answers to fundamental questions regarding segregation of the avian germ line and the molecular basis of pluripotency should foster the full use of avian pluripotent stem cells.

Keywords: Chick; Avian; Embryonic stem cells; Embryonic germ cells; Primordial germ cells; Chimeras

1. Introduction

1.1. Pluripotent stem cell lines in the mouse

The first pluripotent stem cell lines were originally derived from transplantable germ cell tumors or teratocarcinomas, and were complex tumors that consisted of undifferentiated cells among many differentiated cell types derived from the primary germ layers (Martin and Evans, 1975). Stem cell lines isolated from these tumors, known as embryonal carcinomas cells (ECCs), were used as an in vitro model to study the development of the early mouse embryo (Martin, 1980) and in some cases when injected into the blastocyst could participate in the development of a normal adult mouse (Mintz and Illmensee, 1975). However, ECCs where often aneuploid and rarely gave rise to germ cells in chimeric mice after injection into the blastocyst (Illmensee and Mintz, 1976).

The observation that teratocarcinomas often developed after ectopic transplantation of mouse embryos suggested that pluripotent stem cells could be derived from the culture of early embryos. Using techniques similar to that for the culture of ECCs Evans and Kaufman (1981) and Martin (1981) established lines of pluripotent stem cells from cultured mouse blastocysts. This second type of pluripotent stem cell lines, now called embryonic stem cells (ESCs), could be maintained in an undifferentiated state using a mitotically-inactivated feeder layers of embryonic fibroblasts yet retained the ability to form teratocarcinomas upon transplantation, could be induced to differentiate in vitro with a pulse of retinoic acid, could form embryoid bodies, and retained immunological markers in common with the inner cell mass of the blastocyst. Most importantly, the ESCs gave rise to all tissue types including the germ line when injected into recipient blastocysts.

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The standard methods for the establishment and culture of mouse ESCs included the use of embryonic fibroblast feeder layers that provide a developmentally static environment to inhibit the pluripotent epiblast cells from differentiating while permitting proliferation. Subsequently, leukemia inhibitory factor (LIF) was shown to be responsible for maintaining embryonic stem cells in an undifferentiated state and allowed ESCs to be cultured without the use of feeder layers (Nichols et al., 1990; Smith and Hooper, 1987). Other cytokines such as IL-6, oncostatin M, and ciliary neutrophic factor, which act through the gp130 signalling pathway, could also substitute for LIF to maintain mouse embryonic stem cells in an undifferentiated state (Conover et al., 1993; Nichols et al., 1994; Rose et al., 1994; Wolf et al., 1994).

The third class of pluripotent stem cell lines were derived from post-migratory primordial germ cells (PGCs) isolated from the 10.5–11.5 dpc embryo using a combination of feeder layers, LIF, fibroblast growth factor-2, and stem cell factor (SCF) (Matsui et al., 1992; Resnick et al., 1992). Like ESCs, these PGC-derived stem cell lines were able to differentiate in vitro and could give rise to somatic and germ line chimeras when injected into the blastocyst (Matsui et al., 1992). Subsequently, the PGC-derived stem cell lines were called embryonic germ cells (EGCs) to distinguish their origin from ESCs derived from the blastocyst. However, because of abnormal imprinting in EGCs developmental abnormalities could arise in chimeras (Tada et al., 1998).

2. Pluripotent stem cells: the avian perspective

Of the three types of pluripotent stem cell lines in the mouse, the establishment of ESC lines had the most impact on research over the last 20 years. Since, ESCs could be injected into the blastocyst to generate somatic and germ line chimeras (Bradley et al., 1984), they formed the basis for exquisite manipulations of the murine genome using homologous recombination, which has provided a powerful tool to examine gene function during development and in the adult animal (Capecchi, 1989, 2001). In addition, mouse ESCs are used in vitro to study specific developmental processes and in studies to test the molecular basis for cell specification. Because of these advantages, ESCs have been established for other rodents and other mammalian species such as pigs, cattle, and sheep (Prelle et al., 2002), primates (Thomson and Marshall, 1998), and from human embryos (Shamblott et al., 1998; Thomson et al., 1998).

During the last 15 years, those working with the avian embryo have watched with some envy as embryonic stem cell technology has matured to the point where the manipulation of the mouse genome in the form of 'knock-outs' and other derivative manipulations have become almost routine. Nevertheless, a few laboratories have taken the risk of attempting to establish pluripotent embryonic stem cells from the early avian blastoderm or embryonic germ cells from PGCs. The main impetus for the isolation and culture of avian embryonic stem cells has been the hope that such cells could be used to generate transgenic birds with specific modifications to the avian genome, as in the mouse. Therefore, the purpose of this article is to explore the approaches towards the culture of avian stem cells and their current utility.

2.1. Early avian germ line chimeras

One of the wonderful advantages of the avian embryo is its accessibility. Since the avian embryo is self-contained in a calcified eggshell, it lends itself to direct manipulation. This feature has been utilized to produce somatic chimeras of various types through tissue grafts, chorioallantoic grafts, parabiosis, yolk sac chimeras, and neural tube chimeras (Le Douarin and McLaren, 1984). However, in order to utilize any potential avian embryonic stem line for manipulation of the avian genome, it was necessary to develop a reliable means of producing germ line chimeras analogous to that produced by blastocyst injection of murine embryos. Marzullo (1970) was the first to transfer cells from one unincubated chick blastoderm to another and showed that after 14 days of incubation, some of the donor cells from pigmented Rhode Island Red and Barred Plymouth Rock (BPR) breeds were incorporated into non-pigmented White Leghorn (WL) embryos. Unfortunately, none of the embryos survived to hatch. Two decades later, Petitte et al. (1990) demonstrated that this technique could be used to generate germ line chimeras. In this case, dispersed cells from stage X BPR blastoderms were injected into the subgerminal cavity of freshly laid WL embryos. The black pigmentation of the BPR breed is due to homozygosity at the dominant white locus (*I/I*), while the WL line used was homozygous dominant at the dominant white locus (*I/I*). Hence, feather pigmentation of somatic chimeras was readily visible. In addition, DNA fingerprinting could be identified donor BPR DNA in the blood and semen of the chimera. Test mating of the BPR/WL chimera with BPR hens confirmed germ line transmission of the donor cells even though the frequency was low. Since that time, avian germ line chimeras can be produced routinely using blastodermal cell transfer. One of the key features of the process is to 'compromise' the recipient embryo with a sublethal dose of gamma irradiation (Carsience et al., 1993) or through the removal of cells from the area pellucida (Kagami et al., 1997). Both of these techniques allowed the formation of chimeras with high efficiency. The production of somatic and germ line chimeras using early blastodermal cell transfer suggested that it might be possible to culture pluripotent cells from the stage X embryo.

2.2. Avian embryo culture

Another important technique that allows full use of ESCs is ability to culture manipulated embryos to hatch.

Over the last century, various procedures have been developed for the culture of avian embryos. This ranges from New's classic culture method (New, 1955) where unincubated embryos are cultured outside of the egg for a few days to surrogate eggshell systems where the embryo is cultured ex ovo from fertilization to hatch (Perry, 1988). Most techniques are designed for a specific stage of development and for a particular duration (Sellcck, 1996). For the purpose of hatching somatic and germ line chimeras after blastodermal cell transfer, the options are to simply make a window in the egg or to utilize surrogate eggshells.

Recently, Speksnijder and Ivaric (2000) described a windowing technique where the shell is ground off and the opening is covered in phosphate buffered saline before the shell membrane is cut. This permits access for the injection of cells into the embryo but allows no air to enter the egg, which is detrimental to survival. After the injection, the egg is sealed with a piece of moist shell membrane, allowed to dry and covered with Duco® cement. Hatchability improved from about 8% for the usual windowing protocol to about 34%. In addition, Bednarczyk et al. (2000) reported improvements in hatchability after eggs were stored for 5–7 days with the window made in the blunt end of the shell over the air cell.

An alternative to the windowing techniques described above is to use ex ovo culture. This is based upon the procedures of Rowlett and Simkiss (1987); Perry (1988) where two surrogate eggshells are used, one for the first three days of incubation and another where turkey eggshells are used to culture three day chicken eggs. Recently, Borwornpinyo (2000) optimized the hatchability of cultured chicken embryos from freshly laid eggs using surrogate eggshells. The modifications to the original methods of Perry (1988) improved hatchability from the usual 20–50 to over 75%.

Hence, two of the main components for the application of avian ESC technology, the ability to produce somatic and germ line chimeras and the ability to culture these manipulated embryos to hatch have been established for some time. These technological developments were encouraging and provided a framework for using avian ES cells.

3. Avian embryonic stem cells

3.1. Culture methods

Most efforts to culture avian embryonic stem cells have focused on the stage X unincubated blastoderm or PGCs (Fig. 1). The choice of the stage X embryo is a practical one. It is the earliest and the most convenient stage to obtain without having to induce premature oviposition, and somatic/germ line chimeras are easily made using cells of the stage X area pellucida thereby facilitating tests of pluripotency. The next step was to culture pluripotent cells. Etches et al. (1996) cultured whole explanted stage X embryos and dispersed embryo cells in a monolayer, or cultured dispersed cells on a feeder layer of mouse fibroblast for 48 h. Cells from the three culture systems produced somatic chimeras when injected in to stage X recipients but at reduced rate compared to that observed with uncultured cells. The blastodermal cells cultured with mouse embryonic fibroblasts yielded significantly more somatic chimeras than whole explants or dispersed stage X cells cultured alone. Interestingly, contributions to the germ line were observed at an equal frequency regardless of the conditions of culture, but were significantly reduced compared to the frequency and rate of germ line transmission using uncultured cells. Similar short-term culture of stage X blastodermal cells has been reported by Du and Jing (2003).

Pain et al. (1996) cultured blastodermal cells from stage IX–XI chick and stage X–XI quail embryos and reported conditions that allowed for the long-term culture of pluripotent embryonic stem cells. Using alkaline phosphatase as a marker of pluripotency, the best results were obtained with a combination of human LIF, FGF-2, avian or murine SCF, and Il–11 on a feeder layer of inactivated STO fibroblasts. To neutralize any possible induction of differentiation, an antibody against retinoic acid was also added to the media. Like that observed for mouse ESCs, LIF appeared critical to the long-term proliferation and survival of the cultures. In addition, LIF was required to maintain the expression of several markers associated with an embryonic stem cell phenotype, viz. SSEA-1, EMA-1, and EMA-7. Furthermore, telomerase activity was maintained in the avian ESC cultures after multiple passages, but was down-regulated after a pulse of retinoic acid.

In our laboratory, we tested the culture of avian embryonic stem cells using heterologous and homologous feeder layers and conditioned media (Petitte and Yang, 1994; Petitte and Mozdziak, 2002). Dissociated cells from the unincubated chicken blastoderm at stage X were initially cultured with STO feeder layers, primary chick embryonic fibroblast (CEF) feeder layers, or media conditioned by buffalo rat liver (BRL) cells (Smith and Hooper, 1987) or by the chicken hepatocarcinoma line LMH (Kawaguchi et al., 1987). None of these components alone could maintain the blastodermal cells beyond two passages. When the combination of primary CEFs and media conditioned with the LMH cells were used to culture dispersed cells from the area pellucida of the stage X embryo, the cells very quickly differentiated into the primary fibroblast feeder layer. This was unexpected since both primary CEFs and media conditioned with LMH cells are capable of maintaining mouse embryonic stem cells (Yang and Petitte, 1994). In contrast, an STO feeder layer combined with BRL conditioned media maintained chicken blastodermal cells for over 20 passages. Initially, such colonies contained cells heavily laden with lipid droplets, which were eventually lost with subsequent passages. By the third or fourth passage, most cells exhibited an ES-like phenotype, namely, a large

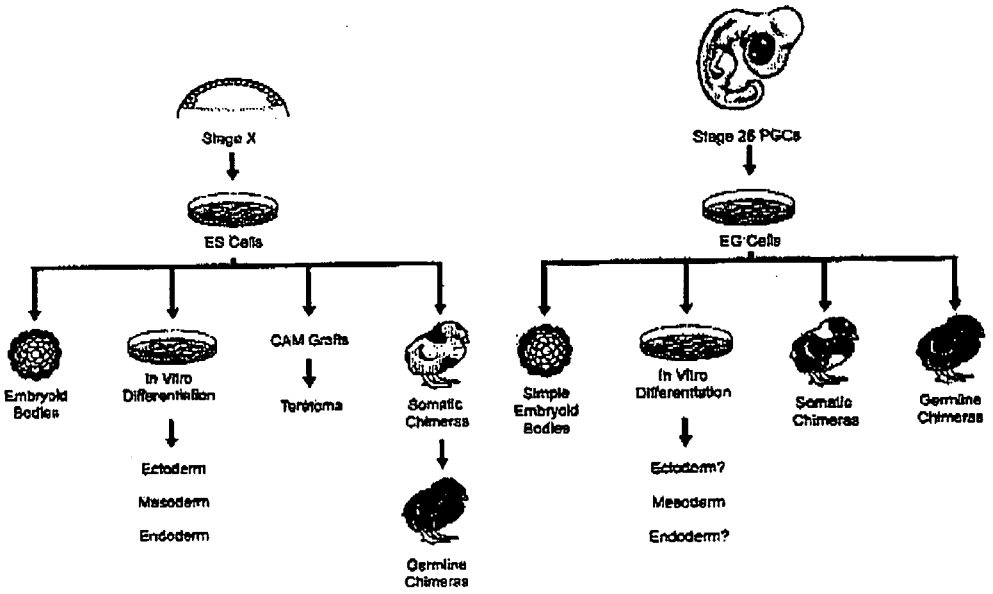

Fig. 1. The origin of embryonic pluripotent stem cells in the chick. Embryonic stem cells established from stage X embryos can form embryoid bodies, differentiate in vitro, and form teratomas when grafted on the CAM. When ESCs are injected into recipient stage X embryos, they can give rise to somatic/germ line chimeras. Primordial germ cells from the stage 27 embryo can be cultured as embryonic germ cells and can form simple embryoid bodies, differentiate in vitro, and form somatic chimeras or germ line chimeras depending upon the stage of the recipient embryo.

translucent nucleus, a prominent single nucleolus, and relative little cytoplasm (Fig. 2). All ES-like cells attached to each other and generally grew in colonies with a well-defined border. Unlike mouse ESCs, the blastodermal cells would invade the STO feeder cells and attach directly to the surface of culture dishes. In a similar manner to that observed by Pain et al. (1996), our chicken ES-like cells expressed SSEA-1 and EMA-1 antigens, which were lost after retinoic acid-induced differentiation (data not shown). Another characteristic of the ESCs is the presence of a single nucleolus rather than two nucleoli, the latter being associated with differentiation of the unicubated embryo (Raveh et al., 1976). Hence, it is possible to culture cells from the stage X blastoderm that maintain an embryonic stem cell-like phenotype for several passages.

The second approach towards the culture of pluripotent avian stem cells has been through the adaptation of the methods used to culture mouse EGCs. Avian PGCs undergo a rather circuitous journey from their localization in the germinal crescent after gastrulation to their final residence in the germinal ridge 72–96 h after incubation (Nieuwkoop and Sutasurya, 1979). During this time frame, it is possible to harvest PGCs from embryonic blood samples at stage 15 or from the undifferentiated gonad in older embryos. Several groups have attempted the short term culture chicken PGCs with the goal of using the cultured cells as a means of developing transgenic birds (Chang et al., 1997, 1995a,b; Ha et al., 2002; Karagenc and Petitte, 2000). With this in mind, Park and Han (2000) cultured chick embryonic germ cells from PGCs harvested from the undifferentiated gonad and established cultures for several months. Dispersed stage 28 gonadal cells, consisting of stroma and germ cells, were cultured in the presence of SCF, LIF, FGF-2, IL-11, and insulin-like growth factor-I (IGF-I). From this initial seeding, the stromal cells appeared to generate a feeder layer to support the growth of the germ cells into colonies. After the first 7 days, the loose colonies of germ cells were passed onto chick embryonic feeder layers that were *not* mitotically inactivated. Unlike avian ESCs or mouse EGCs, the chicken EGCs did not attached to the feeder layer nor could they be maintained using mitotically inactivated STO cells or primary CEFs. The reasons for the latter observation remain unknown. The EG cells were also SSEA-1 positive and periodic acid-Schiff positive, which are indicative of PGCs.

3.2. In vitro differentiation

One of the characteristics of pluripotent stem cells is their ability to differentiate in vitro. Avian ES cells are capable of undergoing differentiation in response to retinoic acid and can form embryoid bodies while cultured as a suspension (Pain et al., 1996). The spontaneous differentiation of avian ESCs included nerve cells, red blood cell

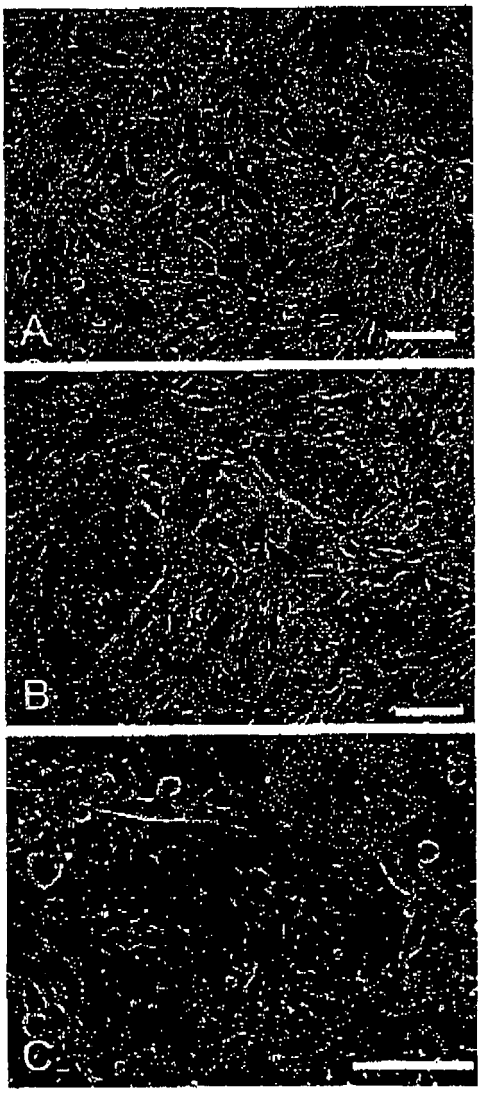

Fig. 2. Phase contrast view of avian embryonic stem cells. Dispersed cells from the area pellucida of stage X embryos were seed onto STO feeder layers and cultured in 80% BRL-conditioned media. (A) One day after seeding, the chick cells have attached to the feeder layer in small clumps with highly refractive lipid droplets. (B) After 7 days of culture, colonies of cells with an ES-like morphology are visible consisting of a large nucleus, little cytoplasm, and prominent nucleolus. (C) High magnification of a colony of ES cells after 11 days of culture. Scale bar, 100 μ.

hematpoiesis and muscle cells (Pain et al., 1996). Likewise, Park and Han (2000) reported that the chicken EGCs could form simple embryoid bodies after removal of the feeder layer and LIF from the media. Other than these two reports,

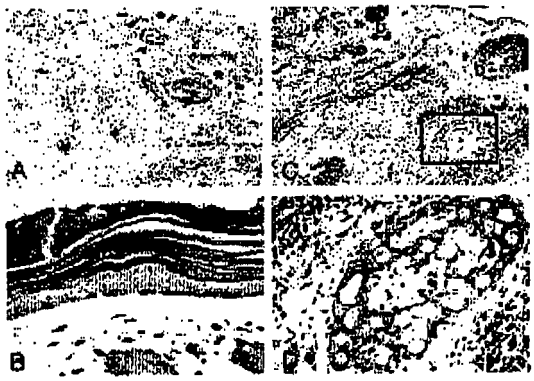

Fig. 3. Histological sections stained with hemotoxylin and eosin of teratoma-like structures obtained after grafting clumps of ES cells on the CAM of 9 day embryos. (A) Low magnification of tumor showing organized epithelial lined lumens (*), regions of hematopoiesis, and fibroblast-like cells. (B) High magnification of a section with a stratified and keratinized surface overlying cells that appear to have secreted a cartilaginous matrix, and loose fibroblast cells. (C) Blood vessels (b) entered the tumor and spread through the fibroblast mesenchyme. (D) High magnification of the box in C showing an unusually organized region. Grafting of whole stage X embryos onto the CAM produced similar teratomas.

in vitro differentiation of avian stem cells has yet to be fully exploited. To assess the pluripotency of our avian embryonic stem cells, we examined their ability to differentiate on the chorioallantoic membrane (CAM) of 9-day chick embryos. Briefly, colonies of avian ESCs cultures exhibiting an embryonic stem cell phenotype were transplanted to the CAM of 9-day embryos. As a control, whole stage X embryos were also grafted to the CAM (McLachlan, 1982). After nine days of incubation, the grafts were removed and sectioned. The under these conditions, the cultured avian ESCs and the whole explanted stage X embryos developed into teratoma-like structures that attempted to form organized tissue (Fig. 3A–D) and stimulated angiogenesis from the CAM into the tumor (Fig. 3D). Regions of hematopoiesis, epithelial-lined lumens of various sizes, cells embedded in a cartilage-like matrix, and highly keratinized cells on the surface of the graft were observed. Similar, though not identical, observations were made after grafting mouse ESC-derived embryoid bodies onto the CAM of the chick embryo (Gajovic and Gruss, 1998).

3.3. *In vivo differentiation*

While cells cultured from the stage X blastoderm may look like ESCs and express markers associated with pluripotency, the most significant test of developmental potential lies in their ability to generate somatic and germ line chimeras. In this regard, Pain et al. (1996) reported that blastodermal cells cultured from 1–3 passages were able to

yield somatic chimeras. These were evaluated using feather color as a marker. In addition, 2 chickens generated from cells cultured for 7 days were found to be germ line chimeras. Park and Han (2000) reported that 4th passage EGC cultures established from WLs gonads were capable of generating feather color chimeras when injected into the Korean Ogol fowl, a bird with black plumage. In addition, PCR-amplified WL-specific DNA was detected in the heart, liver, muscle and gonads of some chicks, suggesting that the cultured cells could give rise to various cell types in vivo. Subsequently, Park et al. (2003) injected WL EGCs into the dorsal aorta stage 17 Ogol embryos. Fluorescent-labeled EGCs from the second passage were found to take up residence in the gonad at 5.5 days of incubation. Subsequent test matings of hatched chicks indicated that the cultured cells gave rise to functional gametes. Apparently, the cultured EGCs functioned more like PGCs and probably were restricted to the original germ cell lineage since similar results were obtained with PGCs cultured for 5 days (Ha et al., 2002).

We examined the ability of cultured ESCs to incorporate into recipient embryos and to give rise to various tissues using mixed-sex chimeras. Chick ESC cultures were established with female embryos, chosen after PCR-screening the area opaca for the presence of W chromosome-specific DNA (Petitte and Kegelmeyer, 1995). Subsquently, colonies of ESCs were individually picked and injected into irradiated recipient unincubated embryos of unknown sex to form chimeras (Carsience et al., 1993). After 10 days of incubation, phenotypic male embryos were screened for the presence of the W chromosome DNA. Fig. 4 shows the presence of female-specific DNA in several tissues from two male embryos, indicating that some of the donor female cells were incorporated into the male embryos. Given this result, we established cultures of avian ESCs from BPR embryos, carefully picked colonies of ESCs and injected them into irradiated donor WL embryos known to be homozygous for dominant white. Somatic chimeras were produced using ESCs cultured up to 8 weeks (Fig. 5). Of these chimeras, 3 males were found to be germ line chimeras (Table 1). Nevertheless, it appeared that the level

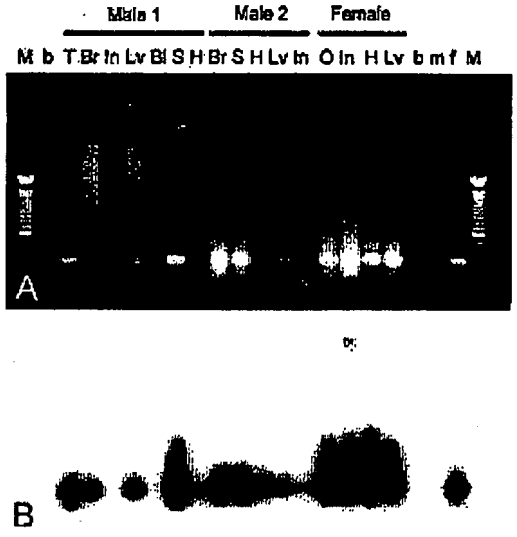

Fig. 4. Detection of W chromosome DNA in two male embryos following the injection of female ESCs into stage X embryos. ESCs cultures were initiated with female stage X cells and cultured for 5 weeks. Colonies were picked and injected into recipient stage X embryos. After 10 days of incubation, phenotypically male embryos were tested for the presence of female DNA in various tissues. (A) PCR amplification of W chromosome-specific DNA from two phenotypically male embryos and a control female embryo. (B) Southern blot of gel in A using a probe internal to the amplification product. For male 1, female DNA was detected in the testis (T), brain (Br), liver (Lv), skin (S) and heart (H), but not in blood (BL) or the intestine (In). In male 2, female DNA was detected in brain, skin, heart, liver and intestine. Control lanes consisted of female embryonic ovary (O), intestine, heart and liver. Other lanes: M, molecular size markers 100 bp ladder; b, water blank; m, purified male DNA; f, purified female DNA.

Fig. 5. Somatic and germ line chimeras generated using chicken ESCs initiated with Barred Plymouth Rock embryos and injected into White Leghorn recipient stage X embryos. (A) Newly hatched feather color chimeric chicks generated with ES cells cultured for 7 days. (B) A mature male from the group that was test mated with barred rock hens and was a germ line chimera.

Table 1
Germ line transmission results of BPR/WL chimeras mated to BPR fowl

Chimera #	Sex	Somatic chimera	Days of ESC culture	White Leghorn chicks (I,I)	Barred Plymouth Rock chicks (J,J)	% Germ line transmission
1841	Male	Yes	7	181	3	1.63
1842	Male	Yes	7	211	1	0.47
1845	Male	Yes	7	179	4	2.18
B386	Male	No	7	211	0	0
1847	Female	No	7	175	0	0
1849	Female	Yes	7	176	0	0
B384	Female	No	7	115	0	0
4530	Male	Yes	9	197	0	0
4528	Male	Yes	9	171	0	0
4532	Male	No	9	177	0	0
4536	Female	No	9	92	0	0
1593	Female	No	20	36	0	0

of feather color chimerism and the frequency of germ line transmission declined with subsequent passages (Table 1). Given that only early passage cells have been able to give rise to germ line chimeras, defining optimal culture conditions to maintain pluripotency is key to using these cells to generate transgenic birds.

4. Genetic modification of pluripotent stem cells

One of the most obvious applications of ESC technology is the ability to genetically engineer changes to the genome to develop transgenic animals. The application of this technology to the modification of the avian genome is important for a number of reasons. First, the chick embryo is a major model of early vertebrate development. The recent sequencing of the chicken genome should foster comparative developmental genetics across other model vertebrate systems. Secondly, targeted modification of commercial poultry stocks using pluripotent stem cells will allow changes to specific loci that would have value for the world-wide production of animal protein in the form of poultry meat. Thirdly, the domestic hen is extremely efficient at protein synthesis during the formation of the egg. This aspect has attracted the attention of the pharmaceutical industries that need a means of producing therapeutic proteins that are post-transcriptionally modified for proper biological function. Hence, strategies to transfect pluripotent stem cells are an important component toward utilizing avian stem cell technology.

Various reports have indicated that fresh blastodermal cells are amenable to transfection using liposomes (Brazolot et al., 1991; Fraser et al., 1993; Rosenblum and Chen, 1995), in ovo electroportation (Muramatsu et al., 1997; Wei et al., 2001), or viral vectors (Bosselman et al., 1989; Salter et al., 1987; Thoraval et al., 1995). With the exception of viral vectors, the efficiency of DNA integration is too low to expect sufficient germ line transmission, even after enrichment of the cells expressing the transgene (Speksnijder, 1996). The culture of pluripotent stem cells should allow the selection of cells that have integrated the transgene and would allow cells to undergo targeted changes to the genome through selection of homologous recombination events (Liu, 1995). In 1999, Pain et al. optimized the transfection of avian ESCs using various liposomes preparations. Typically, the efficiency of cationic liposome mediated transfection is reduced in the presence of serum, and avian ESCs do not survive transfection in the absence of serum (Pain et al., 1999). Using minimal serum concentrations, Pain et al. (1999) observed that differentiated cells were more receptive to transfection then undifferentiated cells, but were able to select undifferentiated ESCs for neomycin resistance. To date, no reports exist using transfected avian ESCs to generate germ line chimeras with transgenic progeny. For these reasons, the major approaches to avian transgenesis has been through retroviral vectors and microinjection of DNA (Sang, 2004).

5. Other applications

Avian pluripotent stem cells can be used for a variety of applications beyond that to generate transgenic birds. Depending upon the procedure used, the formation of somatic chimeras using freshly isolated stage X cells can result in a 'chimera' that is almost visually indistinguishable from the donor phenotype. An interesting prospect would be to culture avian stem cells from genetically superior birds and use the cells to generate high-grade chimeras that would appear to be virtually genetically identical. With adaptations to high-throughput in ovo vaccination technology, it could be possible to generate high-grade somatic chimeras thereby shortening the time needed for conventional poultry breeding programs to generate superior stock (Ricks et al., 2003). In addition, pluripotent avian stem cells could be used for in vitro pharmaceutical applications such as human vaccine development, which often requires the use of embryonated eggs, or in vitro human therapeutic protein production. Finally, embryonic stem cells could be developed from current lines of transgenic chickens that express a reporter gene (Mozdziak et al., 2003a,b), thereby providing stem cells with a cell lineage marker that could be traced in vitro and in vivo.

6. Remaining questions

The lack of any reports, published or otherwise, on the use of avian ESCs for the production of transgenic chickens brings to the fore several questions regarding the relationship between embryonic stem cells and avian PGCs. In the mouse, the pluripotency and self-renewal of embryonic stem cells appears to be maintained through the action of three

transcription factors, Oct-4, Stat3, and Nanog (Chambers et al., 2003; Mitsui et al., 2003). Mouse ESCs require Oct-4 to avoid differentiation into trophectoderm and Nanog to prevent the development of primitive endoderm. Oct-4 and Nanog work in concert with Stat3, activated by LIF/gp130 signaling, to support the maintenance of pluripotency. Elevated expression of Nanog can compensate for the LIF/gp130/Stat3 pathway and can maintain self-renewal of ES cells. Hence, the two transcriptional pathways act independently. Elucidating the molecular basis for pluripotency in other major vertebrate models such as the chick may go a long way toward developing robust methods to culture pluripotent avian stem cells. Unfortunately, attempts to clone avian Oct-4 have been unsuccessful (Soodeen-Karamath and Gibbins, 2001); however, Horiuchi et al. (2004) recently reported the identification of avian LIF. Again, the sequencing of the avian genome and its public availability should go a long way towards understanding the nature of pluripotency in avian stem cells and yield some significant differences compared to mammals.

An additional hypothesis compounds the relationship between embryonic stem cells and germ cells. Recent work on the origin of avian PGCs suggests that the chick could follow the preformed mode of germ cell development. Originally, avian PGCs were thought to arise from the hypoblast (Swift, 1914), then hypoblast/epiblast explants between quail and chick embryos indicated that the epiblast was the source of the germ cells in the germinal crescent (Eyal-Giladi et al., 1981). Subsequently, early germ cells were thought to arise gradually through an epigenetic process beginning with the stage X embryo since germ cells could not be cultured using stage IX embryos, except when placed on an feeder layer of STO cells (Karagenc et al., 1996). However, Tsunekawa et al. (2000) reported that the expression of chicken vasa homolog (CVH) correlated well with the identification PGCs and could be traced back to presumptive PGCs in the early cleavage embryo. Furthermore, vasa protein formed part of a mitochondrial cloud, often associated with preformistic germ plasm in other vertebrates suggesting that avian PGCs could be predetermined in early cleavage embryos. A definitive answer to the preformation vs. epigenetic mechanism of avian germ cell development has crucial implications for the use of blastoderm-derived ESCs for the production of germ line chimeras and transgenic chickens. If avian germ cells are predetermined before ovipoistion in the early cleavage embryo, then it is doubtful that, given current technology, germ cells could arise in vivo from introduced avian embryonic stem cells. Avian EGCs would also have a similar problem when injected into the stage X embryo. In that case, the only route to targeted manipulation of the avian genome is through the culture of PGCs that retain their ability to migrate to the germinal ridge. The recent report of germ line chimeras from EGCs injected into stage 17 embryos argues that these cells are more like PGCs than EGCs (Ha et al., 2002; Park et al., 2003). Fortunately, the question of developmental problems associated with imprinting of the germ line may be relatively minor since imprinting appears to be absent in birds (Nolan et al., 2001).

7. Conclusions

Progress towards the culture of avian pluripotent stem cells has been remarkable given the handful of laboratories working in the field. There have been many attempts to develop pluripotent stem cells in other mammals besides the mouse; however, none have resulted in germline chimeras. On the other hand, chick pluripotent stem cells have resulted in germ line chimeras.

Stem cell biology is a rapidly expanding field that now encompasses not only embryonic stem cells and embryonic germ cells, but small populations of adult stem cells. While the efficiency of germ line chimerism of avian ES and EG cells is low and appears to be best from early passages, as our knowledge of pluripotency expands, so will the potential for using the avian system.

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